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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this study was to 1) identify the key functional angiogenic mediators in the normal and diseased prostate, 2) determine the relationship between disease progression and angiogenic mediators in prostatic fluid; and, 3) determine the efficacy of the natural inhibitor thrombospondin-1 (TSP-1) in treating prostate cancer in model systems. Prostate cells secrete many molecules capable of regulating angiogenesis; however, which are functionally active in angiogenic regulation is unclear. We used an *in vitro* angiogenesis assay to identify the functional angiogenic mediators secreted by prostate cells, and quantified these factors *in vitro* by immunoblot or ELISA and *in vivo* by immunostaining human tissues. Normal prostate epithelial cell secretions were anti-angiogenic due to inhibitory TSP-1 whereas this inhibitor was decreased in the pro-angiogenic secretions derived from benign prostatic hyperplasia (BPH) and cancer cells. This pro-angiogenic activity depended primarily on fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) whose secretion was increased. Immunolocalization confirmed that these changes also occurred *in vivo*. Thus, we identified the major functional angiogenic mediators in the prostate and have provided a detailed description of how normal angio-quiescent prostate tissue becomes angiogenic in disease states through both the down-regulation of the inhibitor TSP-1 and the up-regulation of the stimulatory FGF-2 and VEGF.

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prostate cancer, benign prostatic hyperplasia, Neovascularization, anti-angiogenesis

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Introduction

Prostate cancer is a heterogeneous disease (Cheng et al., 1998; Macintosh et al., 1998) which hampers the development of effective therapies aimed at the tumor cells. The development of anti-angiogenic therapies could circumvent this problem because such therapies target the genetically stable, normal endothelial cells that line the tumor blood vessels. This tumor-induced angiogenesis is necessary for the progressive growth and metastasis of all solid tumors (Folkman, 1971; Bouck et al., 1996; Hanahan & Folkman, 1996). The importance of angiogenesis in prostate cancer is underscored by many studies which report increasing microvessel density as normal tissue progresses to disease states (Wiedner et al., 1993; Fregene et al., 1993; Montironi et al., 1993; Siegal et al., 1995; Sinha et al., 1995; Bostwick et al., 1996; Lissbrandt et al., 1997; Silberman et al., 1997; Mydlo et al., 1998; Borre et al., 1998; Strohmeyer et al., 2000). We set out to 1) identify the key molecules which functionally regulate angiogenesis in the normal and diseased prostate; 2) determine the relationship between disease progression and angiogenic mediator levels in prostatic fluid; and, 3) determine the efficacy of the natural inhibitor thrombospondin-1 (TSP-1) in treating PCa in model systems. The data obtained through the course of these studies have provided a detailed picture of how neovascularization is controlled in the normal prostate tissue, how these controls are breached in proliferative diseases, and has stimulated many new studies in the regulation of angiogenesis in the prostate.

Description of Research Accomplishments and Training to Date

1) Identification of functionally relevant angiogenic mediators in the prostate.

We used the the microvascular endothelial cell migration assay, an *in vitro* angiogenesis assay (Polverini et al., 1991), to measure angiogenic activity in secretions from prostate cells. We collected secretions from short-term cultures of normal prostate epithelial (n=3) and stromal cells (n=2), from benign prostatic epithelial cells (n=6), and cancerous epithelial cells (n=2), as well as from normal epithelial (PrEC) and stromal (PrSC) cell strains and prostate cancer cell lines (DU145, LNCaP, PC-3 and TSU-Pr1). Secretions from short-term cultures of normal prostate epithelial cells inhibited migration of microvascular endothelial cells in the presence of the inducer fibroblast growth factor-2 (FGF-2) (Fig.1A). This was due to the presence of thrombospondin-1 (TSP-1) as neutralizing antibodies to TSP-1 relieved this inhibition (Fig.1A). We confirmed the presence of high levels of inhibitory TSP-1 by Western blot (Fig.1B).

The normal prostate epithelial cell strains (PrEC-18 and -42) secreted less TSP-1 than did our short-term epithelial cultures (Fig.1B, compare 3 μg short-term culture to 10 μg cell strain), secreted higher levels of vascular endothelial growth factor (VEGF) and FGF-2 (data not shown) and induced migration of endothelial cells (Fig.1C & D). However, we noted that the TSP-1 present was functional since the addition of neutralizing antibodies further increased migration (Fig.1C) and the inducers present were the same as in our short-term cultures as tested thus far (data not shown). Therefore, we found the PrEC strains useful for characterization of the functional angiogenic inducers secreted by normal prostate cells. We observed some variability between the two strains, although VEGF and FGF-2 were the dominant functional inducers in both strains (Fig1C). In the PrEC-42 strain interleukin-8 (IL-8), keratinocyte growth factor (KGF/FGF-7) and growth regulated oncogene α (GROα) also contributed to angiogenic induction, while hepatocyte growth factor (HGF) and transforming growth factor β1 (TGF-β1) did not play a role (Fig.1D).

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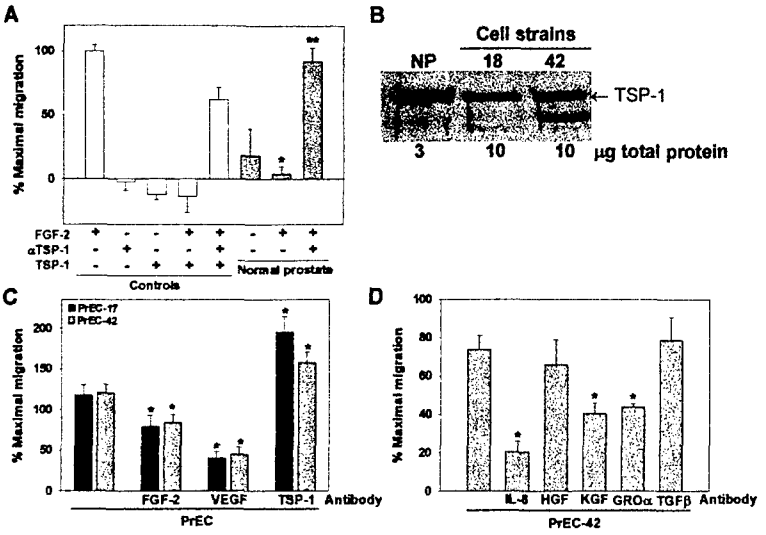

Figure 1. Angiogenic phenotype and mediators present in normal prostate epithelial cells. Serum-free conditioned media were collected from short-term cultures of normal prostate epithelial cells (NP) and two PrEC strains (18 and 42), concentrated and quantified. **(A)** 10 µg/ml total protein from short-term cultures was assayed in a microvascular endothelial cell migration assay alone and in the presence of stimulatory FGF-2 (20 ng/ml) with and without neutralizing anti-TSP-1 antibody (A4.1, produced in our own laboratory, Good et al., 1990). Data is reported as the percent maximal migration toward the positive inducer, FGF-2 (see first bar). **(B)** Protein samples were analyzed by Western blot using the TSP-1 antibody. Lower bands are degradation products of TSP-1 (unpublished observation). Purified TSP-1 protein served as a positive control but is not shown. **(C)** 20 µg/ml total protein from PrEC-18 and -42 were assayed in a microvascular endothelial cell migration assay alone and in the presence of neutralizing antibody to FGF-2, VEGF or TSP-1. **(D)** 20 µg/ml total protein from PrEC-42 was assayed in a microvascular endothelial cell migration assay alone and in the presence of neutralizing antibody to the indicated cytokine. *Indicates that data is statistically significantly different from media alone ($P \leq 0.05$).

In contrast to our normal prostate epithelial cell short-term cultures, secretions from all short-term BPH and cancerous epithelial cell cultures and all four prostate cancer cell lines induced angiogenesis (Fig.2A & B). For each of the cell lines and the cancerous epithelial cell short-term cultures, the ED_{50} dose, the dose of conditioned media at which half the maximal angio-inductive activity is present, was calculated by linear regression analysis of a dose-response curve. The ED_{50} for DU145, LNCaP, PC-3 and TSU-Pr1 was 4, 6, 10 and 12 µg/ml respectively. The ED_{50} for the short-term cancerous epithelial cells was 2 and 7.5 µg/ml. The BPH and cancer short-term cultures continued to express TSP-1, but at decreased levels compared to normal epithelial cells, whereas no TSP-1 was detectable in any of the four prostate cancer cells lines assayed (Fig.2C). In addition, these samples expressed increased levels of the angiogenic inducers FGF-2 and VEGF as compared to normal prostate epithelial cells (Fig.2D & E), and these were identified as the major inducers in both the BPH and cancerous epithelial cell short-term cultures and in the cancer cell lines as the addition of neutralizing antibodies decreased migration (Fig.2A & B). In fact, these molecules appeared to act synergistically in the short-term cancerous epithelial cell cultures (Fig.2B). These data suggest that, in prostate cancer, the transition from an angio-quiescent tissue to an angio-inductive one involves both the down-regulation of inhibitory TSP-1 and up-regulation of inducers VEGF and FGF-2.

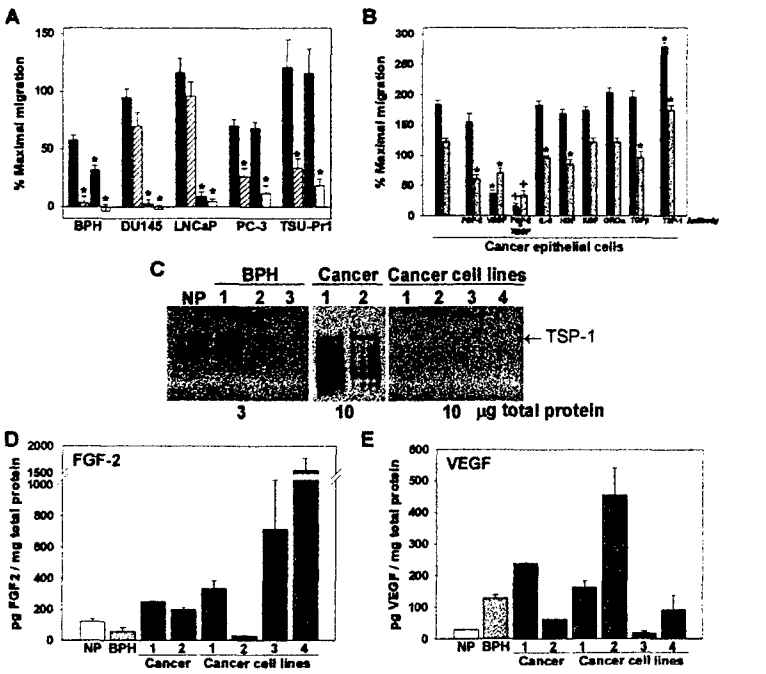

Figure 2. Angiogenic activity and expression of angiogenic mediators in prostate samples. Serum-free conditioned media were collected from a short-term culture of normal prostate epithelial cells (NP), two cultures each of BPH and cancerous epithelial cells and four prostate cancer cell lines. **(A)** 20 µg total protein of each sample was assayed in a microvascular endothelial cell migration assay alone (black bars) and in the presence of neutralizing antibody against FGF-2 (hatched bars), VEGF (gray bars) or both FGF-2 and VEGF (white bars). **(B)** 20 µg total protein of each sample was assayed in a microvascular endothelial cell migration assay alone and with neutralizing antibody against the indicated cytogkine. **(C)** Protein samples were analyzed by Western blot using a TSP-1 antibody A4.1, produced in our own laboratory, (Good et al., 1990). Lower bands are degradation products of TSP-1 (unpublished observation). Purified TSP-1 protein served as a positive control but is not shown. Cell lines are as follows: 1, DU145; 2, LNCAP; 3, PC-3; 4, TSU-Pr1. Total levels of VEGF (D) and FGF-2 (E) were measured in the above samples and in normal prostate epithelial cells (NP) by ELISA (R&D Systems). *Indicates data is statistically significantly different from media alone ($P \leq 0.05$). †Indicates data is statistically significantly different from media alone and from each antibody alone ($P \leq 0.05$).

All samples assayed demonstrated some heterogeneity in the functional angiogenic inducers present. In the prostate cancer cell lines assayed, DU145 and LNCaP relied primarily on VEGF while PC-3 and TSU-Pr1 used primarily FGF-2 (Fig.2A). The BPH specimens also demonstrated heterogeneity with some specimens using FGF-2 and others using both FGF-2 and VEGF (data not shown). These differences were apparent between the two short-term cultures. In addition to VEGF and FGF-2, IL-8, HGF and TGF-β1 contributed to angiogenic activity in only one of the two cultures (Fig.2B). The data for the cancer cell lines are summarized in Table 1. Similar to the short-term cancerous epithelial cell cultures, HGF, IL-8 and TGF-β1 contributed variable angiogenic activity between the cell lines. KGF was not found to be involved in any of the cancer cell lines tested. These data suggest that prostate cancers not only vary histologically and genetically, but also in the angiogenic inducers that they secrete.

Table 1. Summary of active angiogenic inducers secreted by prostate cancer cell lines.

	Relative angiogenic activity associated with each cytokine[a]						
Cell Line	**FGF-2**	**VEGF**	**IL-8**	**HGF**	**KGF**	**GROα**	**TGF-β1**
DU145	-	++++	+	++	-	+	-
LNCaP	-	++++	+	+	-	+	-
PC-3	++	-	++	-	-	-	++
TSU-Pr1	+++	-	-	++	-	-	+++

[a]Relative angiogenic activity was estimated based on data from migration assays testing the ability of neutralizing antibodies against the indicated inducers to reduce the migration of capillary endothelial cells towards the cancer cell conditioned media. Estimated percentage of activity was graded as follows: <20%, -; 20-40%, +; 41-60%, ++; 61-80%, +++; >80%, ++++.

We assayed normal stromal cells for angiogenic activity, using both a short-term culture and a cell strain (PrSC). Contrary to our predictions, both were found to induce angiogenesis in a microvascular endothelial cell migration assay (Fig.3A). VEGF and FGF-2 were active inducers in these samples; however, HGF was the dominant inducer in both samples (Fig.3A). Although again, there were differences between the samples. KGF contributed significantly in the short-term culture sample while TGF-β1 contributed to activity in the PrSC strain. In addition, the short-term culture secreted TSP-1, and this TSP-1 was active, as the addition of neutralizing antibodies to TSP-1 increased migration (Fig.3A & B), while PrSC did not secrete TSP-1 (data not shown). The PrSC strain also secreted higher levels of VEGF and FGF-2 than did the short-term culture (Fig.3C & D). These data suggest that the stromal cells contribute to the overall angiogenic phenotype of the prostate tissue.

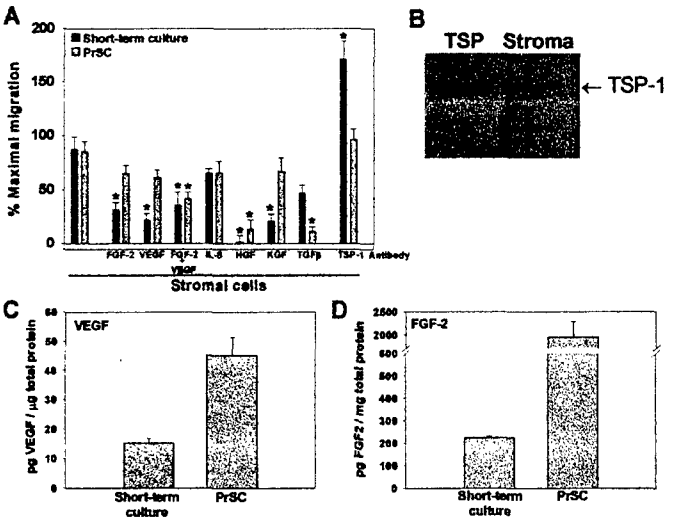

Figure 3. Angiogenic activity and levels of angiogenic mediators in prostate stromal cells. Serum-free conditioned media samples were collected from short-term cultures of normal prostate stromal cells and from a prostate stromal cell strain (purchased from Clonetics, San Diego, CA) and assayed (20 µg total protein) in a microvascular endothelial cell migration assay. Total levels of VEGF (C) and FGF-2 (D) were measured in the above samples by ELISA (R&D Systems). *Indicates that samples were statistically significantly different from media alone (P≤0.05).

To confirm our *in vitro* observations, we immunostained human normal, BPH and cancer tissue for TSP-1, VEGF and FGF-2. The staining intensity was graded in both the epithelial and stromal compartments and compared with the degree of proliferative disease (Fig.4A & B). TSP-1 staining decreased with advanced disease while both VEGF and FGF-2 staining increased, and these trends were evident in both the epithelial and stromal compartments. To determine if loss of TSP-1 correlated with increased angiogenesis, we determined the microvessel density (MVD) and calculated a MVD to TSP-1 staining ratio. When this ratio was plotted against disease state, high grade cancers had a significantly increased ratio as

compared to low grade cancers and BPH (Fig.4C), supporting the role of TSP-1 as a key angiogenesis inhibitor in the normal prostate.

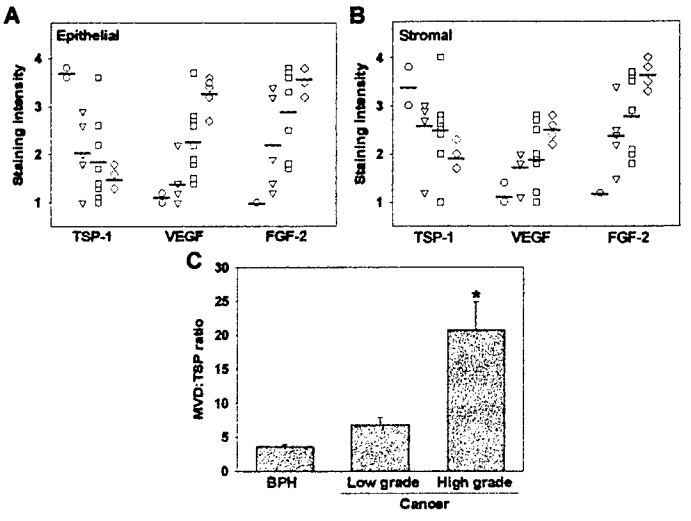

Figure 4. TSP-1, VEGF and FGF-2 expression levels in normal prostate, BPH and prostate cancer tissue. Paraffin-embedded archival prostate tissue was sectioned and immunostained using antibodies to TSP-1, VEGF and FGF-2 and then counterstained with H&E. Relative staining intensity was graded by a pathologist (S.E.C.) as no staining =1; minimal staining =2; moderate staining =3; and intense staining =4. Staining intensity was correlated with disease state in both epithelial (A) and stromal tissues (B). (C) MVD was calculated by counting endothelial lined vessels within tumor regions, and a MVD to TSP-1 staining intensity ratio was calculated and correlated with disease state. *Indicates data is statistically significantly different from BPH and low grade cancer samples ($P<0.05$).

2) Test of relationship between levels of angiogenic mediators in prostatic fluid secretions and prostate disease status.

As reported in our last Annual Summary, prostatic fluid specimens from 9 prostate cancer, 17 BPH, 14 prostatitis, and 3 normal patients were assayed for overall angiogenic activity and for levels of angiogenic mediators. No correlations were observed between patient groups and levels of the angiogenic mediators FGF2, VEGF or TSP-1 or overall angiogenic activity. Thus, we concluded that the measurement of these angiogenic mediator levels in prostatic fluids would not be a clinically useful parameter.

3) Determination of the efficacy of using the endogenous inhibitor in the prostate, TSP-1, to treat prostate cancer in animal models.

Initially, we proposed to transfect prostate cancer cell lines with a TSP-1 expression vector and test *in vivo* tumorigenicity. Thus far, every tumor cell type transfected with a TSP-1 expression vector demonstrated decreased growth, including prostate cancer (Jin et al., 2000). Therefore, we modified our experiment to test the *in vivo* efficacy of TSP-1 treatment, in comparison with anti-VEGF treatment, using a subcutaneous model and the TRAMP model. Several groups have used anti-VEGF treatments against tumors of the DU145 cell line (Borgstrom et al., 1998; Melnyk et al., 1999); however, as we have noted heterogeneity between the cancer cell lines and short-term cancerous cultures, anti-VEGF therapies may not be effective against all prostate cancers.

We tested the neutralizing anti-VEGF antibody we selected for anti-angiogenic activity against purified human VEGF protein in a migration assay (Figure 5A). It inhibited the migration of endothelial cells induced by VEGF, thus demonstrating its effectiveness. We injected nude mice with TSU-Pr1 cells and treated them with either DI-TSP, a peptide mimetic of TSP-1 which has demonstrated potent anti-angiogenic activity in other models (Bouck et al., unpublished data) or an anti-VEGF antibody using i.p. injection. In this model, neither anti-VEGF or the DI-TSP effectively diminished tumor growth. However, although not yet quantified, the DI-TSP treated tumors appeared to have more necrosis. In addition, our collaborators have advised us that they have seen variable results using i.p. injections and that better efficacy is seen using Alzet pumps for a continuous delivery system. Therefore, we plan to repeat these experiments using this method of delivery. We have also

Unpublished data

tried to grow DU145 subcutaneous tumors in nude mice. However, we have not obtained reliable growth of these tumors to allow use in a treatment model. We plan to repeat the DU145 injection using these cells in a matrigel suspension for injection to see if this allows for reliable tumor growth.

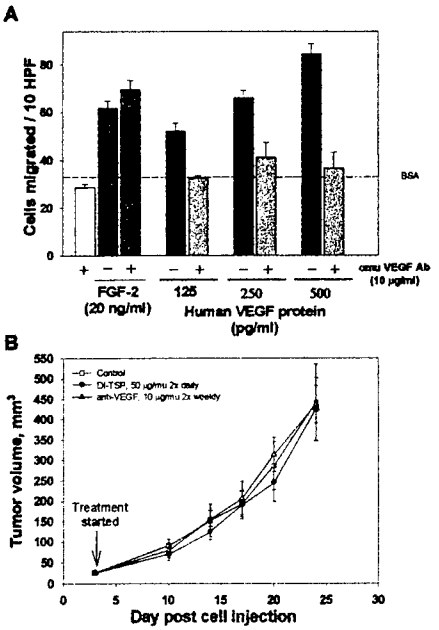

Figure 5. Tests of anti-angiogenic treatment regimens. A) The anti-VEGF antibody was tested for anti-angiogenic activity against human VEGF using the microvascular endothelial cell migration assay. B) 2.5 x 10^5 TSU-Pr1 cells were injected into the right flank of nude mice. Treatment with either DI-TSP, anti-VEGF or vehicle was started on day 3 (5 mice per group). Tumor volume was estimated using the formula [(length x width^2) / 2].

As previously reported, we encountered a long delay in obtaining the TRAMP mice from Jackson Laboratories. We received them on July 24, 2000. We have bred the TRAMP mice to obtain sufficient numbers of male and female TRAMP mice to use for cross-breeding. We planned to cross them with FVB mice to generate F1 TRAMP males for experimentation, as the developer of this model, Norman Greenburg (Baylor College of Medicine) suggested. However, we have had breeding problems with this cross in our facilities. Cross-breeding was initiated in April, and to date, breeding male TRAMP x female FVB mice has generated no pups. In addition, male FVB x female TRAMP crosses have generated only a limited number of litters. Despite these difficulties, we currently have enough male wildtype and TRAMP mice to begin a DI-TSP pilot study when these mice reach sufficient age (~October, 20 weeks of age). For these studies, we plan to use the Alzet pump delivery system as discussed above.

In summary, we have identified the key angiogenic mediators in the normal prostate, BPH and PCa tissue. TSP-1 is the endogenous inhibitor present in the normal prostate that is downregulated or lost during tumor progression. In addition, we have shown that in BPH and prostate cancer the active inducers VEGF and/or FGF-2 are up-regulated. Thus, the transition from normal angio-quiescent prostate tissue to angio-inductive BPH or cancer tissue involves both the down-regulation of the endogenous inhibitor and the up-regulation of underlying inducers.

Summary of Technical Training Received

During the past two years, I have gained significant experience in many areas of basic research. I can now routinely establish short-term epithelial and stromal cell cultures from fresh prostate tissue and culture established cell lines and strains. I am proficient in many *in vitro* assays, including cell viability, proliferation, apoptosis and endothelial cell migration assays, and I have mastered Western blot assays and ELISAs. I have also had extensive exposure to clinical aspects of prostate disease, including histologic examination. In addition, I have gained extensive experience working with mouse models, from learning basic handling and care procedures to autopsy and tissue preparation procedures. I have also become skilled in data analysis, outlining and planing experiments and trouble-shooting problems. In addition, I have gained experience in organizing data for written publications as well as for public presentations.

Key research accomplishments; August 1, 1999 – July 31, 2001

- We identified TSP-1 as the major angiogenesis inhibitor expressed in the normal prostate.

- We have determined that the transition from normal angio-quiescent prostate tissue to BPH and cancer tissue results from down-regulation of TSP-1 along with the up-regulation of VEGF and/or FGF-2.

- We determined that there is marked heterogeneity among angiogenic inducers secreted by prostate cancer cell lines as well as cancer cells from explant tissue cultures, as IL-8, HGF, GROα and TGF-β1 contributed to the overall angio-inductive activity secreted by subsets of these cells.

- We observed that normal stromal cells induce angiogenesis, and that HGF was the predominate inducer, while VEGF and FGF-2 contributed only minor activity.

- We determined that measuring the levels of VEGF, FGF-2 or TSP-1 in prostatic fluids is not a clinically useful indicator of disease state.

- We tested DI-TSP, a TSP-1 peptide mimetic, and anti-VEGF antibody treatment for efficacy against prostate cancer in a subcutaneous TSU-Pr1 cell model in nude mice using i.p. injection and found that neither treatment effectively reduced tumor growth. We intend to re-test the DI-TSP treatment, using a continuous delivery systems as well as testing a combined treatment regimen.

Reportable Outcomes

- Poster presentation at the Keystone Research conference on "Experimental and Clinical Regulation of Angiogenesis," Salt Lake City, Utah, March, 2000. Abstract entitled "PTEN may elicit its tumor suppressor function in part through regulation of angiogenic mediators, " (abstract attached, page 14).

- Poster presentation at the Fall Symposium of the Society for Basic Urologic Research, Fort Myers, Florida, November, 2000. Abstract entitled "Identification of functional angiogenic mediators in the prostate," (abstract attached, page 15).

- Poster presentation at the American Urology Association annual meeting, June, 2001. Abstract entitled :TSP-1, VEGF and FGF2 are key angiogenic mediators in the prostate." Abstract publication reference: Doll et al., The Journal of Urology, 165(5, suppl):51 (abstract attached, page 16).

- Manuscript in press: "Thrombospondin-1, vascular endothelial growth factor and fibroblast growth factor-2 are key functional regulators of angiogenesis in the prostate, " The Prostate (final manuscript submitted attached, pages 17-39).

- Data obtained in these experiments were used as the preliminary data for a grant application submitted to the NIH by Dr. Susan Crawford, Dept. of Pathology, Northwestern University Medical School, the mentor of this grant.

Conclusions

Through the course of these studies, we have identified TSP-1 as the major angiogenesis inhibitor expressed in the normal prostate. In addition, we have shown that the transition from normal angio-quiescent prostate tissue to pro-angiogenic BPH and cancer tissue involves both the down-regulation of inhibitory TSP-1 and the up-regulation of inductive VEGF and FGF-2. These results were seen in both *in vitro* and *in vivo*. In addition, in vitro data indicated that the stromal cells also secrete molecules capable of regulating angiogenesis. Immunostaining revealed that TSP-1 staining decreased with increasing proliferative disease while VEGF and FGF-2 staining increased, and these changes were evident in both the epithelial and stromal tissue. In addition, the decrease in TSP-1 staining correlated with an increase in microvessel density, supporting a key role for this molecule in regulating prostate angiogenesis.

Furthermore, we observed marked heterogeneity between the types and levels of angiogenic inducers secreted, both in established cultures and in short-term cultures of BPH and cancer cells. These data suggest that therapies targeting specific inducers may not be efficacious against all prostate cancers. Thus, the most effective anti-angiogenic treatments against prostate cancer would likely be those targeting the genetically stable microvascular endothelial cells rather than the tumor cells themselves. We began investigating this by testing DI-TSP, a TSP-1 peptide mimetic in a prostate cancer model. However, in our first experiment, using i.p. injection of treatment, we observed no significant effect on tumor growth. We plan to repeat these studies, using a continuous delivery system in subcutaneous models and in the TRAMP model.

The data obtained in these studies have stimulated our investigations into the role of other angiogenesis inhibitors in the prostate and to study the mechanisms of regulation of the key angiogenic mediators, TSP-1, VEGF and FGF-2. For the latter, we are pursuing genetic as well as epigenetic mechanisms of regulation.

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PTEN may elicit its tumor suppressor function in part through regulation of angiogenic mediators.

Doll[1], Jennifer A., Campbell[2], Steve C., Bouck[1], Noël .P. [1]Robert H. Lurie Comprehensive Cancer Center and Dept. Microbiology-Immunology, [2]Dept. Urology, Northwestern University Medical School, Chicago, IL 60611.

The recently identified tumor suppressor gene PTEN encodes a dual specificity phosphatase whose loss activates PIP3 and thereby activates Akt which blocks apoptosis and promotes cell cycle progression. PTEN maps to chromosome 10q, a region that has previously been shown to suppress angiogenesis in glioblastoma cell lines by supporting the production of the anti-angiogenic protein thrombospondin-1 (Hsu et al. Ca.Res. 56:5684-91, 1996). To determine if PTEN is the gene on chromosome 10q responsible for the angiogenic switch, both glioblastoma and prostate cancer cell lines were treated with wortmannin to mimic PTEN effects on PIP3 or were infected with retroviral constructs expressing wild type PTEN, and their secretions were analyzed for angiogenic mediators.

In the glioblastoma cell line U251, restoration of wild type PTEN decreased angiogenic activity as a result of an increase in thrombospondin-1 expression. There was no change in the production of stimulatory VEGF. Wortmannin treatment had similar effects on thrombospondin-1 suggesting that the PIP3 pathway is involved.

The prostate is similar to the brain in that thrombospondin-1 is the major natural inhibitor secreted by normal cells and loss of the PTEN tumor suppressor gene is associated with 50% of advanced tumors. Unlike glioblastomas which uniformly rely on VEGF as their major inducer of angiogenesis, the prostate lines fall into two classes: DU145 and LNCaP rely on VEGF while PC-3 and TSU-Pr1 use primarily bFGF. Prostate tumor lines also differ from glioblastomas in that when those lines mutant for PTEN (LNCaP and PC-3) were treated with wortmannin no change in TSP-1 expression was seen although a dramatic decrease in VEGF did occur. However in these lines introduction of wild type PTEN had no effect on VEGF secretion suggesting that if PTEN is affecting angiogenesis in these lines, it is not doing so via PIP3. These results suggest that in glial cells but not in prostate cells PTEN is capable of controlling angiogenesis and TSP-1 via PIP3 and emphasizes the remarkable tissue specificity of the regulation of key modulators of angiogenesis. (This work was done with support of NIH grants CA52750 CA64239 to N.P.B. and DOD grant DAMD17-99-1-9521 to J.A.D.)

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IDENTIFICATION OF KEY ANGIOGENEIC MEDIATORS IN THE PROSTATE.

Jennifer A. Doll[1], Frank K. Reiher[2], Steve S. Campbell[2], Susan E. Crawford[1,4] and Noel P. Bouck[3,4]. [1]Dept. of Pathology, [2]Dept. of Urology, [3]Dept. of Microbiology-Immunology, [4]Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL.

For growth and metastasis, all tumors require angiogenesis, the growth of new blood vessels from the existing vasculature. In most normal adult tissues, the vasculature is quiescent, an environment maintained in the tissue matrix by a predominance of angiogenesis inhibitors over inducers. In cancer tissue, the secreted levels of inducers and/or inhibitors are altered shifting the balance to favor induction. In prostate cancer (PCa), the changes in secreted angiogenic mediators have not been well characterized. In order to design rational anti-angiogenic treatment strategies, we have identified the key angiogenic mediators in benign prostatic hyperplasia (BPH) and PCa cells. Normal prostate epithelial cells secreted high levels thrombospondin-1 (TSP-1), a potent angiogenesis inhibitor, as observed by Western blot. In a functional angiogenesis assay of endothelial cell migration, normal prostate epithelial cell secretions inhibited migration, and TSP-1 neutralizing antibodies relieved this inhibition indicating that TSP-1 is the major active angiogenic inhibitor present in the normal prostate. TSP-1 secretion was down-regulated in cultured BPH epithelial cells and lost in PCa cell lines (DU145, LNCaP, PC-3 and TSU-Pr1), and secretions from these cells induced migration. In addition to losing TSP-1, both BPH epithelial cells and PCa cell lines secreted elevated levels of vascular endothelial growth factor (VEGF; 4-20 fold) and/or fibroblast growth factor-2 (3-14 fold) which were the dominant angiogenic inducers. Other angiogenic inducers secreted by prostate cells played minor or no role in PCa-induced angiogenesis. Immunostaining revealed strong positivity for VEGF in PCa cells whereas minimal staining for TSP-1 was observed, consistent with our in vitro data. Similar patterns of VEGF and TSP-1 expression were observed in the stroma. Thus, it appears that angiogenesis in the prostate is influenced by both the epithelial and stromal cell secretions. (Supported by US Army grant #DAMD17-99-1-9521).

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TSP-1, VEGF AND FGF2 ARE KEY ANGIOGENIC MEDIATORS IN THE PROSTATE.

Jennifer A. Doll, Frank K. Reiher, Michael Pins, Steve S. Campbell, Noel Bouck, and Susan S. Crawford, Chicago, IL (presented by Dr. Doll)

INTRODUCTION AND OBJECTIVES: Most normal adult tissues maintain an angio-quiescent phenotype by secreting into the tissue matrix a predominance of inhibitors over that of inducers. In disease states, the balance is shifted toward angiogenic induction through alterations in the local levels of the secreted molecules by both tumor and stromal cells. The changes in angiogenic factors are not well characterized in prostate disease. In this study, we have identified the key angiogenic mediators in the normal prostate, benign prostatic hyperplasia (BPH) and cancer.

METHODS: Serum-free conditioned medium collected from short-term cultures of normal epithelial and stromal cells, BPH and cancerous epithelial cells; epithelial and stromal cell strains (Clonetics, San Diego, CA), and PCa cell lines DU145, LNCaP, PC-3 and TSU-Pr1 was assessed for angiogenic activity using the microvessel endothelial cell migration assay alone and in the presence of neutralizing antibodies to vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2) and other angiogenic inducers. Protein levels were measured by Western blot analysis or ELISA, and immunohistochemistry (IHC) was performed on formalin fixed paraffin-embedded archival specimens.

RESULTS: Normal prostate epithelial cells were angio-inhibitory, and this was due to high levels of secreted thrombospondin-1 (TSP-1), a potent angiogenic inhibitor, as neutralizing antibodies to TSP-1 relieved the inhibition. In contrast, normal stroma, BPH and cancer cells were angio-inductive. VEGF and FGF2 were the predominant inducers whereas most other inducers tested contributed minor activity. TSP-1 secretion was down-regulated or lost in BPH and cancer cells, while VEGF and/or FGF2 secretion were increased. IHC on archival prostate tissues confirmed these observations, and also demonstrated that these changes were observed in both the epithelial and stromal cells.

CONCLUSIONS: The transition from an angio-quiescent normal prostate to an angio-inductive phenotype in BPH and cancer involves the up-regulation of the angiogenic inducers VEGF and/or FGF2, and the down-regulation of the angiogenesis inhibitor TSP-1 in both the epithelial and stromal cells.

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Thrombospondin-1, Vascular Endothelial Growth Factor and Fibroblast Growth Factor-2 are Key Functional Regulators of Angiogenesis in the Prostate

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ABSTRACT

BACKGROUND. Prostate cells secrete many molecules capable of regulating angiogenesis; however, which of these actually function as essential regulators of neovascularization is not yet clear.

METHODS. Functional angiogenic mediators secreted by normal and diseased prostate cells were identified using an *in vitro* angiogenesis assay. These factors were quantified by immunoblot or ELISA and localized in tissue by immunohistochemistry.

RESULTS. Normal prostate epithelial cell secretions were anti-angiogenic due to inhibitory thrombospondin (TSP-1) whereas this inhibitor was decreased in the pro-angiogenic secretions derived from benign prostatic hyperplasia (BPH) and cancer cells. This pro-angiogenic activity depended primarily on fibroblast growth factor-2 (FGF-2) and/or vascular endothelial growth factor (VEGF) whose secretion was increased. Immunolocalization studies confirmed that the changes detected *in vitro* also occurred *in vivo*.

CONCLUSIONS. During disease progression in the prostate, production of TSP-1, the major inhibitor, is down-regulated while that of stimulatory FGF-2 and/or VEGF rise, leading to the induction of the new vessels necessary to support tumor growth.

KEY WORDS. prostate cancer, benign prostatic hyperplasia, neovascularization, anti-angiogenesis, prostate stroma

INTRODUCTION

Prostate cancer is an extremely heterogeneous disease where multiple foci of cancer within the same individual often vary in Gleason grade and underlying genetic alterations [1,2]. Such heterogeneity among tumors hampers development of effective therapeutic interventions aimed at tumor cells. One way to circumvent this problem is to target instead the genetically stable normal endothelial cells that form the vessels essential for progressive growth and metastasis of all tumors [3-5]. The importance of angiogenesis in prostate cancer is underscored by the many studies which report increasing microvessel density as normal tissue progresses to disease states [6-10], and by a positive correlation of vessel density with disease-specific parameters and outcome [11-16]. Understanding the molecular mechanisms underlying angiogenesis in prostate diseases may lead to the development of more effective therapies aimed at the vasculature of the diseased prostate.

Angiogenesis is regulated by the balance between the multiple stimulating and inhibiting mediators that are secreted into the extracellular environment [4,5]. In most normal adult tissues, vessels are quiescent due to the presence of high levels of inhibitors and low levels of inducers. For both physiological and pathological angiogenesis, the levels of secreted mediators shift so inducers predominate thus stimulating the adjacent vasculature. In cancers, this shift is often a consequence of the genetic and epigenetic alterations that drive tumor progression resulting in either a decrease in secreted levels of inhibitor(s), an increase in secreted levels of inducer(s), or a combination of both [4,17].

In different tissues, different angiogenic mediators can predominate [18,19]. Several potent inducers of angiogenesis are expressed by prostate epithelial and stromal cells [20-22], and many of them are significantly increased in disease states. Increased expression of vascular endothelial growth factor (VEGF) has been observed in benign prostatic hyperplasia (BPH) [23] and in prostate cancer [23-25]. In cancer tissue, VEGF levels correlate with increasing Gleason score [26,27], tumor stage [28], and disease-specific survival [29]. Several studies also demonstrate that VEGF expression is androgen regulated in the prostate [30-33]. In addition, neutralizing antibody to VEGF can inhibit growth of DU145 tumors *in vivo* [34,35]. However, additional inducers seem likely to be involved in prostate cancers for the highly metastatic subline of the PC-3 prostate cancer cell line, PC-3M, actually secretes less VEGF than does the less aggressive DU145 cell line [36,37]. A wide variety of additional proteins that are also capable of inducing angiogenesis have been found to be expressed in prostatic disease, including fibroblast growth factor-2 (FGF-2) [38,39], interleukin-8 (IL-8) [40,41], hepatocyte growth factor (HGF/scatter factor) [22], keratinocyte growth factor (KGF/FGF-7) [39,42], growth regulated oncogene α (GROα) [41] and transforming growth factor-β1 (TGF-β1) [43,44]. But these molecules have other activities in addition to their ability to influence angiogenesis, and these activities can be tissue and cell-type specific, thus which of these molecules are actually functioning to regulate neovascularization in the prostate is uncertain.

Several known angiogenic inhibitors are also associated with the prostate. Thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis [45,46], is expressed in normal prostatic tissue and variable expression patterns are reported in a small study of cancers [47]. However, its functional relevance to angiogenesis is unknown since TSP-1 can have other biological activities, from activating TGF-β [48,49] to mediating adhesion [50]. TSP-1 is known to be controlled by p53 in some tissues [51,52], and Mehta et al. [53] recently correlated mutant p53 staining with

decreased TSP-1 and increased angiogenesis in prostate tumor tissue, suggesting that TSP-1 could be a key inhibitor of prostate angiogenesis.

The importance of other angiogenic inhibitors in the prostate is unclear. Prostate cancer cell lines PC-3, DU145 and LNCaP secrete activity capable of cleaving plasminogen to form the anti-angiogenic factor angiostatin [54], and PC-3 tumors can inhibit the growth of distant metastases in mice [55]. But, it has not been demonstrated whether this systemic effect is due to angiostatin or to one of the many other inhibitors that can hold distant metastases in check [56,57].

Here we have asked which of the many angiogenic mediators known to be present in the prostate actually play key functional roles in the regulation of its vasculature. Secretions of normal human epithelial cells and stromal cells and of BPH and cancer cells were collected and an *in vitro* assay was used to identify the functionally important inducers and inhibitors of angiogenesis. Then relative *in vivo* levels of crucial mediators were estimated in a variety of prostate tissues by immunohistochemistry. The results provide a detailed picture of how neovascularization is controlled in the normal tissue of this organ and how these controls are breached in proliferative diseases.

MATERIALS AND METHODS

Cell culture. Prostate cancer cell lines, DU145, LNCaP, PC-3 (American Type Culture Collection) and TSU-Pr1 (a generous gift from Chung Lee, Northwestern University, Chicago, Illinois) were maintained in DMEM (Gibco BRL, Grand Island, NY), 10% fetal calf serum, and 2 mM glutamine and antibiotics. Normal prostate epithelial cell strains (PrEC-17, -18 and -42 isolated from 17, 18 and 42 year old donors, respectively) and a normal stromal cell strain (PrSC; Clonetics, San Diego, CA) were maintained according to Clonetic's protocols.

For prostate epithelial cell isolation, we defined normal tissue as specimens from 15-20 year old donors which limited our sample size. Normal prostate tissue (three donors, 17-18 years old), BPH (n=7) and cancerous tissues (n=2) were obtained from Northwestern Memorial Hospital (Chicago, IL). Stromal cells were isolated from histologically normal tissue of a radical prostatectomy specimen (confirmed by M.R.P.). Minced tissue was plated into 10 cm dishes, and media replaced daily. Cell out-growths were seen after 7-10 days, and cells were passaged at ~80% confluency. Conditioned medium was collected from normal, BPH and cancerous epithelial cell cultures at passage one and from stromal cells at passages 2-5. After initial studies demonstrated decreased TSP-1 with passages 4 and 5, only passage ≤ 3 were used for studies. Specific cell types were enriched by growth in cell type-specific media (Clonetics) and success confirmed by morphology. Greater than 90% of cells of the selected cell type was considered adequate for study. All cells were grown at 37 °C with 5% CO_2. Serum-free media were conditioned for 24-48 hours, concentrated, and dialyzed against PBS using a 5 kDa cutoff filter (Millipore, Beverly, MA), and total protein concentrations determined using Coomassie dye binding assays (Pierce, Rockford, IL).

Bovine adrenal capillary endothelial cells (a gift from Judah Folkman, Children's Hospital, Harvard Medical School, Boston, MA) were maintained in DMEM, 10% donor calf serum, 2 mM glutamine, 100 μg/ml endothelial cell mitogen (R&D Systems, Minneapolis, MN) and antibiotics at 37 °C with 7% CO_2 and used at passage 14-15. Human microvascular endothelial cells (Clonetics) were maintained in endothelial cell growth medium (Clonetics) at 37 °C in 5% CO_2 and used at passage 3-12.

Proteins and antibodies. TSP-1 protein was isolated as described [49,57]. Neutralizing TSP-1 antibody (A4.1) was prepared from murine ascites fluid [45] or purchased (Lab Vision, Freemont, CA). Other proteins and neutralizing antibodies were obtained from R&D Systems. Prior to use in migration assays, antibodies were dialyzed against PBS to remove azide and other contaminants. The following concentrations of proteins and antibodies were used in migration assays: VEGF, 100-200 pg/ml; FGF-2, 15-20 ng/ml; IL-8, 100 ng/ml; HGF, 10 ng/ml; KGF, 10 ng/ml, GROα, 100 ng/ml; TGF-β, 1-2 pg/ml; TSP-1, 5 nM; αVEGF, 20 μg/ml; αFGF-2, 10 μg/ml; αIL-8, 40 μg/ml; αHGF, 40 μg/ml; αKGF, 40 μg/ml; αGROα, 40 μg/ml; αTGF-β, 20 μg/ml; and, αTSP-1, 40 μg/ml.

***In vitro* angiogenesis assay**. The capillary endothelial cell migration assay was performed essentially as previously described [58]. Briefly, the procedure is as follows. Capillary endothelial cells were plated in the lower wells of Boyden chambers at 1-1.5 x 10^6 cells/ml, the chamber inverted and incubated for 1.5-2 hours at 37 °C, 5% CO_2 to allow cell attachment to a gelatinized Nuclepore membrane (5 μm pores; Fisher, Pittsburgh, PA). Test substances were added to the top wells, incubated an additional 3-4 hours, and chambers disassembled.

Membranes were fixed, stained, mounted on slides, and the number of cells that had migrated to the upper side of the membrane were counted (ten high power fields per well). Samples were assayed in quadruplicate, and data confirmed by at least two independent experiments. Serum-free medium containing 0.1% BSA served as a control for background migration, and the angiogenic inducers FGF-2 or VEGF served as positive controls. Results were presented as a percent of the maximal migration toward the positive control after subtracting the background migration (serum-free media with 0.1% BSA). Controls demonstrating that proteins and neutralizing antibodies had the expected activity were included in all assays but not always shown.

Angiogenic activity in conditioned media was determined by assaying a range of concentrations (0-200 μg/ml) and calculating the protein concentration at which half the maximal migration was achieved (ED_{50}) using a linear regression of the dose curve. The ED_{50} was used for comparison between samples. For neutralizing antibody studies, all conditioned media samples were tested at 20 μg/ml except for media from donor-derived normal prostate epithelial cells that were tested at 10 μg/ml due to limited material.

Protein expression. Levels of secreted VEGF and FGF-2 were quantified by ELISA (R&D Systems) per manufacturer's instructions. TSP-1 expression was determined by immunoblot analysis of conditioned media proteins resolved by SDS-PAGE (8%), electro-blotted to Hybond-C membrane (Amersham, Arlington Heights, IL), probed with α-TSP-1 antibody, developed by chemiluminescence (Amersham), and exposed to x-ray film. The Coomassie dye binding assay was used to confirm equal protein loading between samples.

Immunohistochemistry (IHC). Paraffin-embedded formalin-fixed tissue sections were subjected to microwave antigen retrieval and stained for TSP-1, VEGF or FGF-2 using an Avidin-Biotin-Peroxidase protocol. Antibodies detecting VEGF (R&D Systems) and FGF-2 (American Diagnostica Inc., Greenwich, CT) and the TSP-1 A4.1 antibody were used for staining. For TSP-1, endothelial cell staining served as an internal positive control [19]. For VEGF and FGF-2, tissues known to express these molecules were used as positive controls. BSA used instead of the primary antibody served as the negative control. The intensity of brown color was graded by a pathologist (S.E.C.) blinded to the identity of the stain or sample (1 = no staining noted; 2 = focal, mild staining; 3 = multifocal, moderate staining; 4 = diffuse, intense staining), and specific cell types identified by morphology. Tissue sections were immunostained for microvessel density with a CD31 antibody (Pharmingen, San Diego, CA) using a Vectastain elite kit (Vector Laboratories, Inc., Burlingame, CA) and a microwave antigen retrieval step. Microvessel density was calculated by counting CD31 positive endothelial-lined vessels in five non-overlapping random high power fields (40x) within intratumoral regions. One representative section of prostate tissue from each sample was assessed by a pathologist (S.E.C.) in a blinded fashion.

Statistical analysis. Statictical significance of data was determined by using Student t-tests. P values of less than 0.05 were considered statistically significant.

RESULTS

TSP-1 is the key angiogenic inhibitor secreted by the normal prostate epithelium. Normal prostate epithelial cells were cultured in epithelial cell-specific media from tissue obtained from young male donors (17-18 years old). The resulting cell out-growth was composed of >90% epithelial cells based on morphology. The presence of a small number of other cell types was not considered a confounding problem as, in the *in vivo* environment, these cells would also contribute to the local balance of angiogenesis inhibitors and inducers present in the extracellular matrix. We collected serum-free conditioned medium from the first passage of the above cultures of normal prostate epithelial cells (n=3). Angiogenic activity present was assessed using the microvascular endothelial cell migration assay, an *in vitro* angiogenesis assay. Over many years of testing samples in both the migration assay using capillary endothelial cells and the cornea assay for neovascularization, the activity observed in the *in vitro* migration assay has always predicted accurately whether or not a sample would induce neovascularization in the cornea *in vivo* [18,19,45,46,59,60]. The media alone from the short-term normal prostate epithelial cell cultures did not induce migration of the endothelial cells, and when combined with stimulatory FGF-2, it inhibited the migration (Fig. 1A). This anti-angiogenic activity was due to the presence of TSP-1 as the addition of neutralizing antibodies to TSP-1 relieved the inhibition and revealed underlying positive angiogenic activity (Fig. 1A). Western analysis confirmed the presence of high levels of TSP-1 in the normal prostate epithelial cell secretions (Fig. 1B). In this figure, the upper band represents the 180 kDa TSP-1 monomer while the lower band(s) a degradation product that is also anti-angiogenic (unpublished observations and [46]).

Three commercially obtained normal prostate epithelial cell strains (PrEC-17, -18, -42), that had been grown more extensively in culture than our short-term cells, behaved differently in that they were stimulatory in a migration assay (Fig. 1C and D; data not shown) as reported by others for a PrEC strain [20]. All three cell strains did secrete TSP-1; however, the levels were much lower than the short-term epithelial cells (Fig. 1B; data not shown). As the addition of neutralizing antibodies to TSP-1 did further increase migration, the TSP-1 secreted by these strains was active; however, it was insufficient to suppress angiogenic activity, thus revealing the underlying inductive activity (Fig. 1C). Therefore, as more conditioned media could be collected from the cell strains than from the short-term cultures, we used the media collected from two of these cultures (PrEC-17 and -42) with a panel of neutralizing antibodies to identify the key angiogenic inducers secreted by normal prostate epithelial cells. Both VEGF and FGF-2 contributed to the angiogenic activity secreted by these strains (Fig. 1C; data not shown). These were the only functional inducers identified in secretions of PrEC-17 whereas IL-8, KGF and GROα also contributed significantly to the angiogenic activity of the PrEC-42 strain (Fig. 1D).

BPH secretions were angiogenic due mainly to VEGF and/or FGF-2.

In contrast to the inhibitory nature of secretions of short-term normal epithelial cell cultures, secretions from seven similar epithelial cell cultures derived from BPH tissue all induced angiogenesis over a range of doses (Fig. 2A; data not shown). The ED_{50}, the dose at which half-maximal activity was achieved, was calculated as an estimate of overall angiogenic activity for two different BPH cultures and was 10 μg/ml. FGF-2 and VEGF accounted for the majority of the angiogenic activity in these secretions, as neutralizing antibodies to these factors significantly reduced activity (Fig. 2A). When absolute amounts of VEGF and FGF-2 present in the cellular secretions were measured, as expected from the functional assays, BPH cells produced an

average of 4.5-fold more VEGF than normal prostate epithelial cells but no more FGF-2 (Fig. 2B and C). TSP-1 was secreted at high levels by some but not all of these cultures (Fig. 2D), suggesting that down-regulation of TSP-1 secretion may play an essential role in the development of angiogenesis in BPH.

Cancer cell secretions were angiogenic due mainly to VEGF and/or FGF-2.

Secretions of two short-term cultures of cancer epithelial cells and four cancer cell lines all induced angiogenesis over a wide range of concentrations (Fig. 2A and E; data not shown). The ED_{50} for the cancer cell lines DU145, LNCaP, PC-3 and TSU-PR1 were 4, 6, 10 and 12 μg/ml, respectively. The calculated ED_{50} for the two short-term cancer cultures were 2 and 7.5 μg/ml.

We used a panel of neutralizing antibodies to identify the molecules responsible for the majority of the angiogenic activity, and in all cases VEGF and/or FGF-2 were the predominant inducers (Fig. 2A and E). In the two short-term cancer epithelial cell cultures, VEGF and FGF-2 were both involved in angio-inductive activity and seemed to have a synergistic effect (Fig. 2E), as has been described previously for these molecules [61,62]. In one of the cultures, IL-8, HGF and TGF-β also contributed (Fig. 2E). When permanent cell lines were tested similarly, they varied significantly from one another. (Fig. 2A; Table I). DU145 and LNCaP depended most heavily on VEGF for their angiogenic activity; whereas, FGF-2 was the primary inducer secreted by PC-3 and TSU-Pr1. Other factors played variable roles (Table I). As expected from the functional data, both the short-term cultures of cancers and three of the four cancer cell lines secreted VEGF at levels from 2.2 to 16-fold higher than those secreted by cultures of normal prostate epithelial cells (Fig. 2B). FGF-2 levels were increased 1.7 to 13-fold over normal epithelial in three of the four cancer cell lines (Fig. 2C).

None of the cancer cell lines expressed TSP-1 (Fig. 2D) although LNCaP cells occasionally secreted very low levels (data not shown), possibly related to the reported TSP-1 promoter methylation in this cell line [63]. Both short-term cultures of cancer cells secreted minimal levels of TSP-1 (Fig. 2D). The amount of TSP-1 was evidently not sufficient to overcome the high level of inducers present in these secretions, and despite its obvious degradation (Fig. 2D), this TSP-1 retained some activity because when it was neutralized, angiogenic activity increased (Fig. 2E). Although the degradation of TSP-1 may be an artifact of the culture conditions or conditioned media collection process, previous studies by our lab and others have demonstrated that common proteolytic fragments of TSP-1 retain anti-angiogenic activity [46].

Stromal cells contribute to the angiogenic phenotype in the normal prostate.

We collected serum-free conditioned media from a short-term culture of normal stromal cells and also from a commercially obtained stromal cell strain (PrSC) to characterize the angiogenic phenotype of normal prostate stromal cells. Secretions of all cultures induced the migration of capillary endothelial cells (Fig. 3A), but secretions of the commercial strain were more active than those of the short-term culture with ED_{50}'s of 10 and 15 μg/ml, respectively (data not shown). Only the short-term stromal cell culture secreted TSP-1 in amounts detectable by Western analysis (Fig. 3B; data not shown). This inhibitor played a role in dampening the secreted angiogenic activity for when it was neutralized, activity increased (Fig. 3A). However, the production of TSP-1 by stromal cells was apparently lost with increasing passage (data not shown), suggesting that only early passage cells ($p \leq 3$) were suitable for characterization; thus, only conditioned media collected from passage 3 or under were used in the functional studies.

As VEGF and FGF-2 were the predominant inducers secreted by tumor epithelial cells, we investigated the secreted levels of these molecules in stromal cells. Short-term cultures of stromal cells secreted very little VEGF compared to the normal epithelial cells and higher levels of FGF-2 than normal epithelial cells (Figs. 2B,C and 3C,D). For both molecules, the PrSC strain secreted higher levels than the short-term stromal culture (Fig. 3C and D). The addition of neutralizing antibodies against angiogenic inducers to conditioned media revealed that HGF was the dominant inducer secreted by stromal cells while FGF-2 and VEGF contributed only minor activity (Fig. 3A). In addition, KGF and TGF-β contributed some activity to the short-term culture and to the PrSC strain respectively (Fig. 3A).

Immunostaining supported key roles for TSP-1, VEGF and FGF-2 in prostate angiogenesis.

We used IHC to stain prostate tissue sections from normal tissue (n=2), BPH (n= 5), low grade cancers (Gleason score 2-6; n=11), and high grade cancers (Gleason score 7-10, n= 5) for TSP-1, VEGF and FGF-2 protein expression (Fig. 4). We defined normal prostate as tissue specimens from 15-20 year old donors, thus, limiting our sample size. TSP-1 localized strongly to the stroma and moderately to the epithelium in normal prostate tissue (Fig. 4A). The staining of this inhibitor of angiogenesis progressively decreased when normal epithelial tissue was compared to BPH and cancer specimens and when low-grade cancers were compared to high-grade cancers (Fig. 4A-D, M). Reduced TSP-1 expression was especially evident in the stroma of cancer tissue (Fig. 4, A-D, N), where decreased expression correlated with progressive disease.

VEGF staining was minimal to absent in normal prostate tissue, but showed a stepwise increase in both epithelial and stromal cells in BPH and cancer (Fig. 4E-H, M and N). Likewise, FGF-2 staining was minimal to absent in normal tissue and increased in both BPH and cancer in both epithelial and stromal cells (Fig. 4I-N). In addition, a progressive increase was seen in both inducers when low-grade cancers were compared to high-grade ones (Fig. 4G, H, K-N). As observed in the functional studies, production of VEGF and FGF-2 *in vivo* varied between cancer specimens (Fig. 4M and N, and compare Fig. 4, G to H, K to L) confirming the heterogeneity within this disease.

To determine if the intensity of TSP-1 staining correlated with microvessel density, we calculated a ratio of microvessel density to TSP-1 staining in these samples. The microvessel density increased with advanced proliferative disease with mean values (± standard error) of 9.1 ± 0.3, 10.0 ± 1.8, and 30.13 ± 4.4 in BPH, low grade cancer and high grade cancer, respectively. Only the high grade samples were statistically significantly different from the BPH and low grade cancer samples ($P<0.02$). The ratio of microvessel density to TSP-1 staining also increased with advanced proliferative disease, from 3.61 ± 0.3, 6.79 ± 1.0 and 20.79 ± 4.1 in BPH, low grade cancer and high grade cancer, respectively (Fig. 4O). For the microvessel density to TSP-1 staining ratio, the high grade cancers were significantly different from both the BPH and low grade cancer samples (Fig. 4O; $P<0.02$), suggesting that decreased TSP-1 expression is associated with increased neovascularization.

DISCUSSION

We identified TSP-1 as a key functional inhibitor of angiogenesis in normal prostate tissue. Data presented here identified TSP-1 as responsible for the anti-angiogenic activity of normal prostate epithelial cells. TSP-1 was also secreted in an active form by normal stromal cells. Although TSP-1 secretion decreased and was eventually lost with increasing passage in culture (passages ≥4), as also happens in cultures of BPH epithelial cells and fetal astrocytes (unpublished observations), immunostaining of normal prostate tissue showed it to be highly expressed *in vivo* in both epithelial and stromal compartments. The importance of TSP-1 in regulating prostate angiogenesis was underscored by our observations that in short-term cultures of epithelial cells of BPH and cancer origin and in four prostate cancer cells lines, secreted TSP-1 levels were decreased or absent, and that *in vivo*, TSP-1 down regulation was in proportion to the progression of proliferative disease. In the rat prostate, TSP-1 expression decreases with aging [64]. Although such a natural decline in TSP-1 expression with age has not been reported in the human prostate, if it does occur, it could contribute to progression of cancer with advancing age. These data suggest that, in prostate disease, loss of TSP-1 contributes to the permissive angiogenic environment that is essential for epithelial hyperplasia or for small nests of tumor cells to begin to grow progressively.

Numerous molecules capable of inducing angiogenesis are present in the prostate. In normal epithelial tissue we and others [27] have observed occasional low levels of VEGF *in vivo* and in secretions of short-term cultures, although the high level of TSP-1 in such secretions ensures that their overall activity is to inhibit angiogenesis. In cancerous tissues VEGF and/or FGF-2 were consistently the major active inducers present. However, HGF and IL-8 each contributed to angiogenesis in 1 out of the 2 short-term cancer epithelial cell cultures and 3 out of the 4 cancer cell lines, suggesting that some heterogeneity exists in the active inducers present between cancer specimens.

Although BPH is also a proliferative disease and urine from patients with BPH can induce endothelial cell proliferation [65], ours is the first direct demonstration that BPH cells themselves are indeed angiogenic. Secretions of short-term cultures derived from BPH were angiogenic due in part to increased production of VEGF and FGF-2 and decreased TSP-1 expression. Immunohistochemical stains were concordant with the altered expression patterns in BPH and cancers demonstrating progressive dysregulation of these angiogenic mediators with increasing proliferative disease suggesting that our *in vitro* data accurately reflects the *in vivo* situation.

We also observed that the prostate stroma, which is known to play a crucial role in development and maintenance of gland integrity [22], also contributed to the control of angiogenesis. Stromal cells from normal tissue produced anti-angiogenic TSP-1 *in vitro* and *in vivo* and its level fell with increasingly severe proliferative disease. Stromal production of angiogenic inducers increased in parallel with the severity of the proliferative disease. This data is in keeping with that from other tumor types in which tumor stroma can undergo a transition and begin to produce angiogenic factors [66]. A similar role has been proposed before for prostate-tumor-associated stromal cells [67]. Such cells have been shown by others to be able to stimulate the growth of prostate tumor cells *in vivo* [68,69], but ours is the first direct evidence that this effect may be, in part, mediated through stromal regulation of angiogenesis.

We show here that prostate tumors, known to be genetically heterogeneous [1,2], are also angiogenically heterogeneous. The latter trait is likely to be a consequence of the genetic differences as the secretion of angiogenic mediators is often regulated by oncogenes and tumor

suppressor genes [4,17]. In different short-term cultures of tumor epithelial cells and cancer cell lines the relative importance of different angiogenic factors varied widely, with some tumors heavily dependent on FGF-2 whereas others relied primarily on VEGF. These *in vitro* findings seem to accurately reflect the situation *in vivo*. Our *in vitro* data indicating that DU145 relies on VEGF and PC-3 on FGF-2 is consistent with the *in vivo* findings of Connolly and Rose [37] that demonstrate orthotopic DU145 tumors grown in nude mice produce three times as much VEGF as similarly grown PC-3M tumors which, in turn, produced higher levels of FGF-2. This kind of angiogenic heterogeneity suggests that if anti-angiogenic therapies targeting specific inducers are used to treat prostate cancer, inconsistent results might be expected. Even if the major *in vivo* inducer is identified for each patient, our observation that all prostate tumors produced multiple angiogenic factors raises the possibility that disabling one angiogenic inducer may simply select for those tumor cells producing higher amounts of another one. However, many anti-angiogenic agents target the endothelial cells, making them insensitive to multiple angiogenic inducers; thus, it is possible that both BPH and prostate cancers can be effectively controlled by such agents. One such approach would be to replace the natural inhibitor in the prostate, TSP-1, which has proven effective in other cancer models (Reiher et al., manuscript submitted). The usefulness of such therapy in prostate cancer is strongly supported by Jin et al. [70] who demonstrate that the growth of DU145 tumors in Balb/c mice is inhibited by overexpression of TSP-1. We are currently investigating the use of TSP-1 protein therapy in prostate cancer models.

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Table I. Summary of active angiogenic inducers secreted by prostate cancer cell lines.

	Relative angiogenic activity associated with each cytokine[a]						
Cell Line	**FGF-2**	**VEGF**	**IL-8**	**HGF**	**KGF**	**GROα**	**TGFβ**
DU145	–	++++	+	++	–	+	–
LNCaP	–	++++	+	+	–	+	–
PC-3	++	–	++	–	–	–	++
TSU-Pr1	+++	–	–	++	–	–	+++

[a]Relative activity was estimated based on data from migration assays testing the ability of neutralizing antibodies against the indicated inducers to reduce the migration of capillary endothelial cells towards the cancer cell conditioned media. Estimated percentage of activity was graded as follows: <20%, –; 20-40%, +; 41-60%, ++; 61-80%, +++; >80%, ++++.

Figure Legends

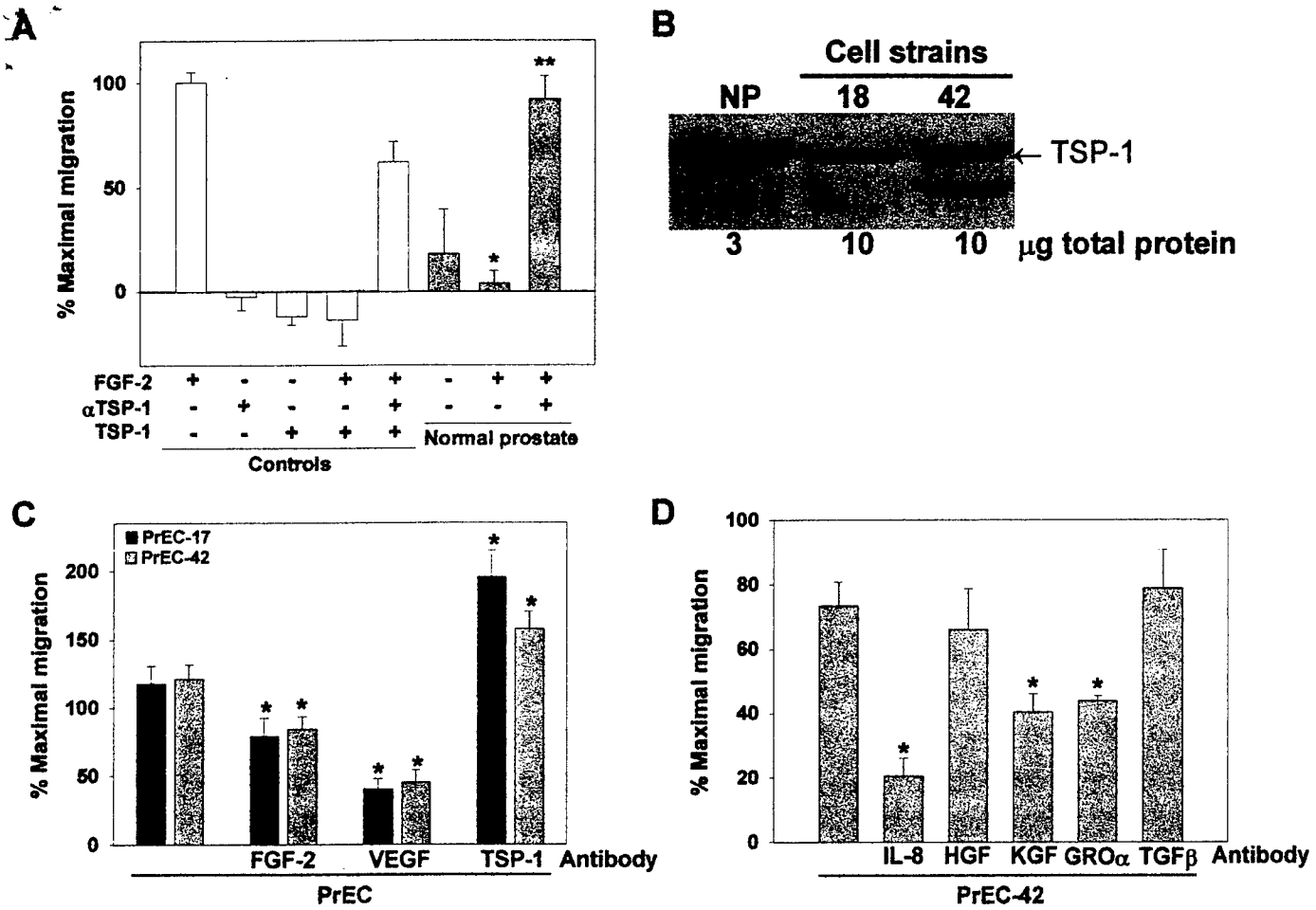
Fig. 1. Normal prostate epithelial cells are anti-angiogenic due to TSP-1. (**A**) Serum-free conditioned medium from short-term cultures of normal human prostate epithelial cells was tested in a microvascular endothelial cell migration assay for ability to inhibit endothelial cell migration toward an angiogenesis inducer (FGF-2) in the presence and in the absence of antibodies that neutralize TSP-1 (αTSP-1). 100% migration equals the migration toward the positive control, FGF-2 (see first bar). Statistically significant differences ($P \leq 0.05$) were observed when normal prostate media with FGF-2 was compared to FGF-2 alone (*) and when normal prostate media with FGF-2 was compared to the same sample with neutralizing antibody to TSP-1 (**). (**B**) Western blot of conditioned media from short-term cultures of normal prostate epithelial cells (NP) and normal epithelial cell strains PrEC-18 and 42 developed with αTSP-1 antibody A4.1. (**C**) Conditioned medium from PrEC-17 and -42 was tested in a microvascular endothelial cell migration assay alone (two far left bars) or in the presence of antibodies that neutralize stimulatory VEGF or FGF-2 or inhibitory TSP-1. Bars are as follows: black, PrEC-17; gray, PrEC-42. (**D**) Conditioned medium from PrEC-42 was tested in a microvascular endothelial cell migration assay alone (far left bar) or in the presence of neutralizing antibodies against angiogenesis inducers IL-8, HGF, KGF, GROα and TGFβ. For (**C**) and (**D**), statistically significant differences between the test media alone and samples with neutralizing antibodies are indicated with an * ($P \leq 0.05$).

Fig. 2. Epithelial cells from BPH and cancer are angiogenic due to VEGF and FGF-2. (**A**) Conditioned medium collected from short-term BPH epithelial cell cultures (BPH) and from prostate cancer lines (DU145, LNCaP, PC-3 and TSU-Pr1) was tested in a microvascular endothelial cell migration assay alone (black bars) and in the presence antibodies that neutralize inducers FGF-2 (hatched bars), VEGF (gray bars) or both VEGF and FGF-2 (white bars). 100% migration equals the migration toward the positive control, FGF-2. Levels of VEGF (**B**) and FGF-2 (**C**) were measured by ELISA in media conditioned by short-term cultures of normal epithelial cells, BPH or cancer epithelial cells (two samples) and by cancer cell lines denoted by numbers (1 = DU145; 2 = LNCaP; 3=PC-3; 4=TSU-Pr1). (**D**) Western analysis of media conditioned by short-term cultures of normal prostate (NP, 3 μg protein), BPH (three samples, 10 μg protein each) or cancer epithelial cells (two samples, 10 μg protein each) or by prostate cancer cell lines (20 μg each) developed with TSP-1 antibody A4.1. (**E**) Conditioned media collected from cultures of two short-term cancer epithelial cell cultures (sample 1, black bar; sample 2, gray bar) were tested in a migration assay alone (two far left bars) and with a panel antibodies that neutralize the indicated inducer or inhibitor of angiogenesis. 100% migration equals the migration toward the positive control, FGF-2. An * indicates that the samples were statistically significantly different from the test media alone ($P \leq 0.05$). A (+) indicates that the samples were statistically significantly different from the test media alone and from the test media with either antibody alone ($P \leq 0.05$).

Fig. 3. Prostate stromal cells secrete angiogenic mediators. (**A**) Serum-free conditioned media collected from short-term cultures of stromal cells (black bars) and from a stromal cell strain (PrSC; gray bars) were tested in a migration assay alone (two far left bars) or in the presence of neutralizing antibodies against the indicated angiogenesis inducer or the inhibitor TSP-1. 100% migration equals the migration toward the positive control, FGF-2. Samples that are statistically significantly different from the test media alone are indicated with an * ($P \leq 0.05$). (**B**) Western

analysis of media conditioned by short-term cultures of stromal cells at passage 3 developed with anti-TSP-1 antibody A4.1. Purified TSP, shown in the first lane, served as a positive control. Levels of angiogenic inducers VEGF (**C**) and FGF-2 (**D**) present in media conditioned by primary normal stromal cells and stromal cell strain PrSC were measured by ELISA.

Fig. 4. Expression of angiogenic mediators in human tissue. (**A-L**) Archival specimens from normal prostate, BPH, low and high grade prostate cancer were immunohistochemically stained for TSP-1 (**A-D**), VEGF (**E-H**) or FGF-2 (**I-L**) and counterstained with hematoxylin. Representative sections are shown. Staining was graded separately in the glandular epithelium (**M**) and in the stroma (**N**) as negative (1), mild (2), moderate (3) or strong (4) by evaluation of 5 random fields per slide, and the grades were averaged and graphed by tissue type. Bars indicate the mean staining value (grades 1-4). Symbols for **M** and **N** are as follows: normal prostate (○); BPH (▽); low grade cancer, Gleason score 2-6 (□); high grade cancer, Gleason score 7-10 (◇). (**O**) The ratio of microvessel density to TSP-1 staining was calculated for each sample. An * indicates that the high grade cancers were statistically significantly different from both the BPH and low grade cancer samples ($P<0.02$).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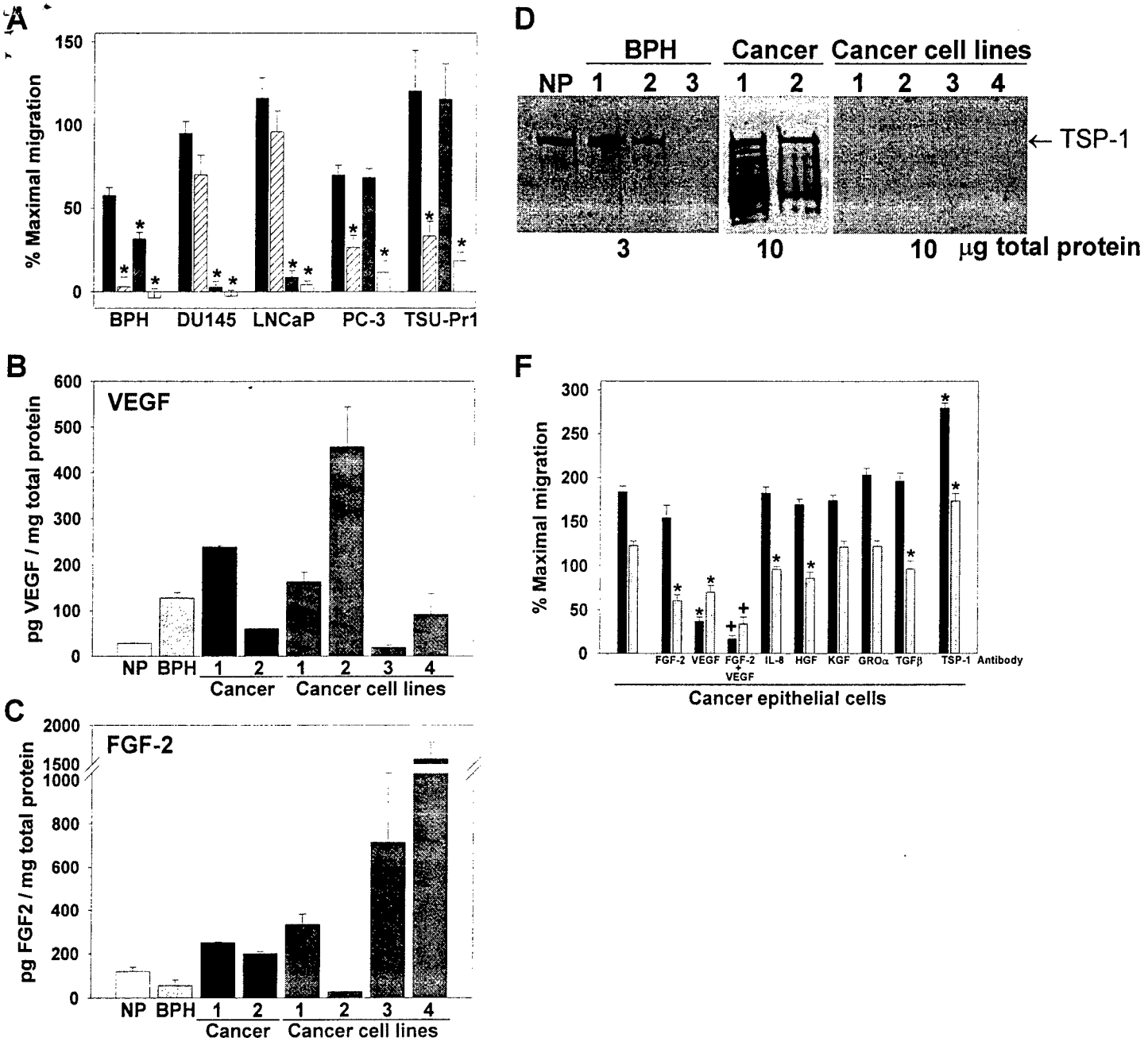
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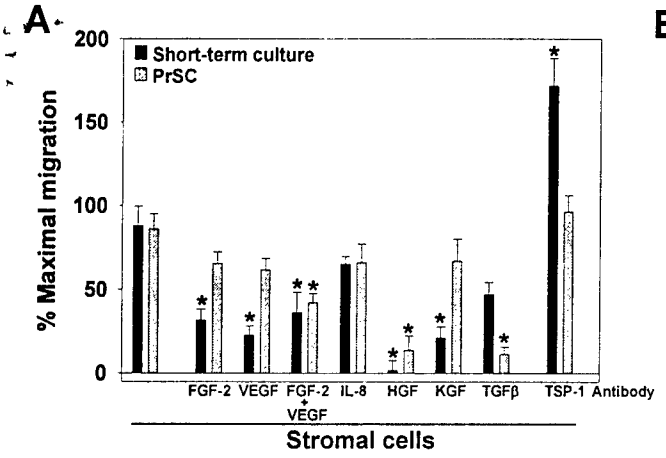
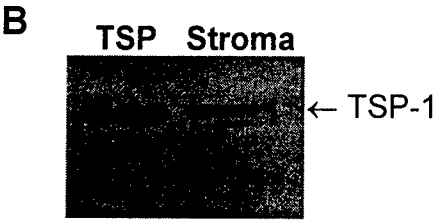
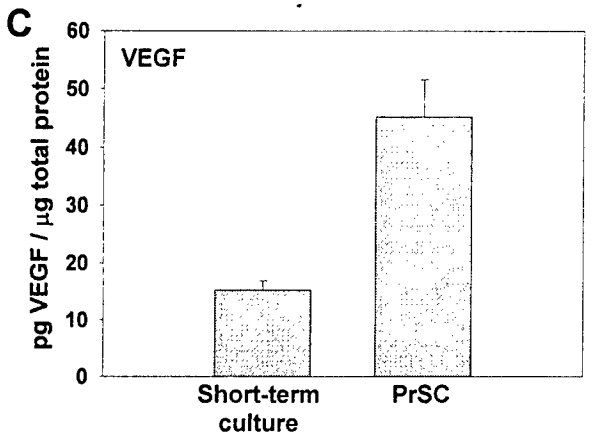
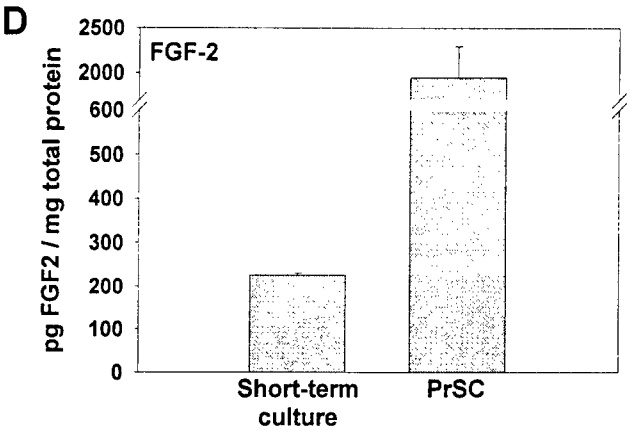
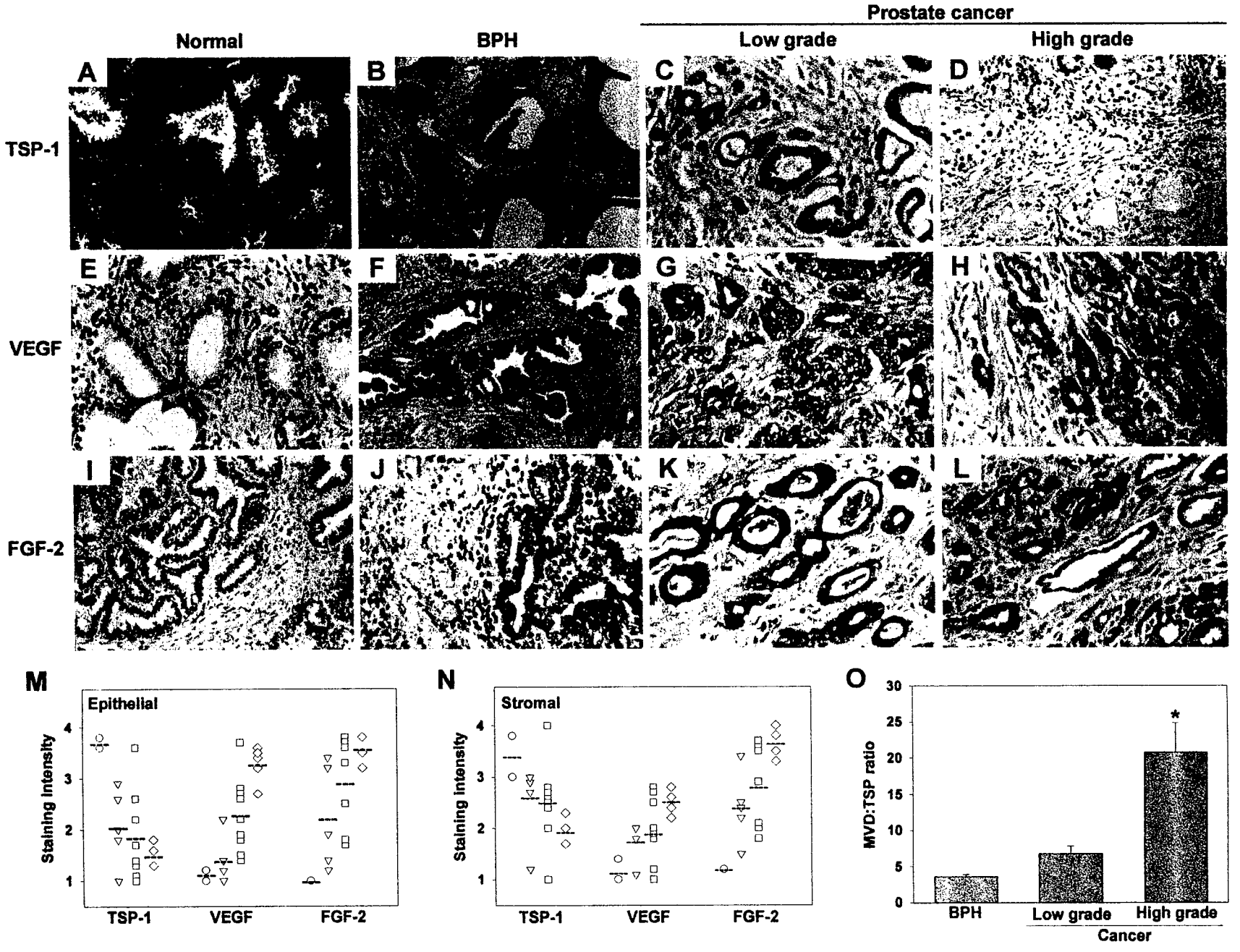
FGF-2
VEGF
FGF-2 + VEGF
IL-8
HGF
KGF
GROα
TGFβ
TSP-1
Antibody
Cancer epithelial cells


A
200
150
100
50
0
% Maximal migration
Short-term culture
PrSC
FGF-2
VEGF
FGF-2 + VEGF
IL-8
HGF
KGF
TGFβ
TSP-1
Antibody
Stromal cells


B
TSP
Stroma
← TSP-1


C
VEGF
pg VEGF / µg total protein
60
50
40
30
20
10
0
Short-term culture
PrSC


D
FGF-2
pg FGF2 / mg total protein
2500
2000
600
500
400
300
200
100
0
Short-term culture
PrSC


Prostate cancer
Normal
BPH
Low grade
High grade
A
B
C
D
TSP-1
E
F
G
H
VEGF
I
J
K
L
FGF-2
M
Epithelial
Staining intensity
4
3
2
1
TSP-1
VEGF
FGF-2
N
Stromal
Staining intensity
4
3
2
1
TSP-1
VEGF
FGF-2
O
MVD:TSP ratio
30
25
20
15
10
5
0
*
BPH
Low grade
High grade
Cancer

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 11 Mar 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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